



Isolation of a Puumala-like Virus from *Mus musculus* Captured in Yugoslavia and Its Association with Severe Hemorrhagic Fever with Renal Syndrome

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An outbreak of severe hemorrhagic fever with renal syndrome (HFRS) occurred in 1988 in Pozarevac, Serbia, Yugoslavia. The disease was diagnosed in 4 children and 1 adult, and 1 of the children died. Rodents were captured from the same area and virus isolation attempted. A hantavirus, POZ-M1, was isolated from lung tissues of hantavirus antigen-positive *Mus musculus*. Serology and restriction enzyme digestion of polymerase chain reaction-amplified segments from this virus showed that it was a strain of Puumala (PUU) virus, the causative agent of nephropathia epidemica. While *Clethrionomys glareolus* is the major rodent host for PUU virus, these results suggest that *M. musculus* may also play an important role in harboring and transmitting PUU-like viruses. The serologic association of this virus with patients with severe HFRS reaffirms that PUU-like viruses may cause severe disease in addition to the generally mild form normally associated with nephropathia epidemica.

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Hemorrhagic fever with renal syndrome (HFRS) is an infectious viral disease caused by viruses in the family Bunyaviridae, genus *Hantavirus* [1, 2]. The reservoirs of hantaviruses are rodents and other small mammals, and transmission to humans is primarily by inhalation of aerosolized excreta [3]. The first serologically confirmed case of HFRS in Serbia was reported in 1979 [4], although cases were recorded elsewhere in Yugoslavia as early as 1952 [5]. HFRS appears in both mild and severe forms in Yugoslavia, with a mortality rate of 5%–16% [6], and the number of recorded cases has been increasing. Hantavirus antibodies or antigens (or both) were previously detected in at least 11 species and subspecies of small mammals captured in HFRS-endemic regions of Yugoslavia [7, 8].

We report summaries of clinical histories of patients seen during an outbreak of severe HFRS in Pozarevac, a small town 60 km southeast of Belgrade. Serologic results from patients' paired sera and the isolation and characterization of a strain of Puumala (PUU) virus, POZ-M1, recovered from lung tissues of mice (*Mus musculus*) captured in and around the houses of these patients are also reported.

Materials and Methods

Capturing and processing of rodents. Small mammals were live-trapped in February and October 1988 in and around houses of people affected with HFRS. Captured animals were identified to species, and lung tissues were removed aseptically and stored in liquid nitrogen for transport, then at -60°C . Lung tissues from 37 small mammals were sectioned ($4\ \mu\text{m}$) by cryostat and examined for hantavirus antigens by indirect fluorescent antibody test (IFA) [9] with convalescent sera from patients with Korean hemorrhagic fever or nephropathia epidemica. Rodent sera, diluted twofold from 1:16 in 0.01 M PBS, were tested for evidence of hantavirus infection by IFA on Vero E-6 cells infected with Hantaan (HTN) virus (strain 76-118). Sera with specific fluorescence at a dilution $\geq 1:32$ were considered positive.

Virus isolation and cell culture adaptation. Lung tissue of antigen-positive mice and rats were pooled in groups of 3 (mice) or tested individually (rats); 10% suspensions (wt/vol) were made in Earle's MEM containing 10% heat-inactivated fetal bovine serum, 2% penicillin/streptomycin, and 0.1% amphotericin B (Life Tech, Grand Island, NY), transferred to a sterile bag, blended for 5 min in a Stomacher mechanical blender (model 80; Tekmar, Cincinnati), and transferred to sterile 15-mL centrifuge tubes. Larger tissue fragments were allowed to settle for 2–5 min. Supernatants were used to inoculate adult rats, newborn rats, and Vero E-6 cells. Five female, 6-week-old Wistar-Furth rats (Charles River Laboratories, Wilmington, MA) known to be free of anti-hantavirus antibodies were injected (0.5 mL) intramuscularly. Newborn Wistar-Furth rats were inoculated intracranially (ic) and intraperitoneally (ip) with 0.03 mL, while subconfluent monolayers of Vero E-6 cells were inoculated with 2 mL of lung suspension. Flasks were kept at 37°C for 1 h. 5 mL of maintenance medium was added to each flask, and cultures were incubated for 14 days at 37°C . Animals were observed for 28 days, blood was taken by cardiac puncture, and sera were screened for anti-hantavirus antibody by IFA [9].

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Lungs of adult rats and brains of newborn rats were examined for hantavirus antigen by IFA as an index of viral replication. Vero E-6 cells were subcultured at 14-day intervals. An aliquot of cells was placed on 10-well spot slides and examined for hantavirus antigen by IFA with immune rabbit sera specific for HTN and PUU (strain P360) [2] viruses and convalescent serum from a patient from Pozarevac. Serial passages were continued at 14-day intervals to 56 days. Antigen-positive cell cultures were used for polymerase chain reaction (PCR) amplification with restriction endonuclease analysis and for comparison by cross-IFA [10], cross-EIA [11], and cross-plaque-reduction neutralization tests (PRN) [10].

Characterization of POZ-M1. POZ-M1, isolated from mice, was compared with HTN, PUU, Seoul (strain HR80-39), Prospect Hill (strain Wisconsin), and Thailand (strain Thai749) viruses by cross-IFA and cross-EIA. Sera were serially diluted two-fold from 1:40 to 1:2560 for IFA and 1:100 to 1:6400 for EIA. For cross-PRNT, serotype-specific antisera from immune rats and convalescent sera from a patient with HFRS from Pozarevac were serially diluted twofold from 1:20 to 1:1024. PRN titers were expressed as the reciprocal of the highest serum dilution producing $\geq 80\%$ reduction of plaques visualized by neutral red or immunoperoxidase stains.

Reverse transcriptase (RT) PCR. Vero E6 cells infected with POZ-M1 were pelleted and lysed in 4 M guanidine isothiocyanate solution. The lysate was layered onto a 5.7 M cesium chloride cushion and subjected to ultracentrifugation at 170,000 g for 21 h in an SW41 Ti rotor for isolation of total cellular RNA [12]. RT-PCR was done with hantavirus-reactive primer pairs for amplification from both M and S genome segments. HG2F1/HG2R1 amplified a 365-bp fragment from the G2 protein-encoding region of the M segment [2], and PUU-S4/PUU-S6 amplified a 280-bp fragment from the S genomic segment [13]. Amplified products from both M and S segments were digested with selected restriction endonucleases and restriction patterns compared with those of an established data base [2, 13].

Results

Outbreak report. In February 1988, an outbreak of severe HFRS occurred in a family in Pozarevac. Four children of this family, aged 2–12 years, became ill within 1 week. Fulminant disease was observed in the 2-year-old, who died 1 day after hospital admission with sudden onset of cardiac failure and pneumonia. Autopsy revealed submucosal and subserosal hemorrhage; hyperplasia of lymphoid tissue in the spleen, liver, and mesenteric lymph nodes; bilateral interstitial pneumonia; and kidney tubular necrosis [14]. The other 3 children, aged 5, 8, and 12 years, were ill simultaneously, with predominant symptoms of fatigue, fever, myalgia, edema, sore throat, headache, gastrointestinal illness, arterial hypertension, exanthema, hematuria, proteinuria, increased urine creatinine, melena, lymphadenopathy, and hepatosplenomegaly. The preliminary diagnoses were acute renal insufficiency and suspected hemorrhagic fever. IFA on these patients' paired sera indicated recent hantavirus infections. Tests for anti-leptospiral antibody were negative. Tests on

Table 1. Cross-assays of POZ-M1 and different hantavirus antigens with immune rat or human sera: immunofluorescent antibody (IFA), EIA, and plaque-reduction neutralization (PRN).

Antigens	Antisera					
	POZ-M1	HTN	SEO	PUU	PH	THAI
IFA						
POZ-M1	>2560	640	640	1280	320	160
HTN	40	>2560	>2560	40	<40	160
SEO	<40	2560	>2560	40	<40	640
PUU	2560	40	40	>2560	80	40
PH	80	40	<40	40	>2560	40
THAI	40	320	320	40	40	640
EIA						
POZ-M1	1600	100	400	1600	100	100
HTN	<100	>6400	>6400	<100	<100	>6400
SEO	<100	400	>6400	<100	<100	100
PUU	400	400	1600	>6400	100	400
PH	<100	<100	100	<100	1600	<100
THAI	<100	>6400	>6400	100	<100	>6400
PRN (80% reduction titers)						
POZ-M1	>1024	20	>1024*	1024		
HTN	<20	>1024	20*	<20		
PUU	1024	20	1024*	>1024		

NOTE. POZ-M1, local hantavirus isolate from *Mus musculus* captured in outbreak area; HTN, prototype Hantaan virus, strain 76-118; SEO, Seoul virus strain HR80-39; PUU, Puumala virus strain P360; PH, Prospect Hill virus, strain Wisconsin; THAI, Thailand virus, strain 749. Titers are expressed as reciprocals of highest serum dilution yielding positive reactions.

* Convalescent serum sample from 46-year-old patient.

serum from another child not living in the house during the outbreak were negative for anti-hantavirus antibody.

In July 1988, a 46-year-old man residing in the same neighborhood developed severe HFRS with fever, vomiting digested blood ("coffee-ground" vomitus), diarrhea, diplopia, conjunctival injection, back pain, rash, hematoma, hepatomegaly, hematuria, proteinuria, increased blood urea nitrogen, evidence of electrolyte abnormalities (hypocalcemia, hyperphosphatemia, and hyperkalemia), and anuria. He experienced all five phases of severe HFRS and was admitted in shock but survived after hemodialysis and prolonged hospitalization. Serology revealed neutralizing antibody with highest titers to PUU virus and the POZ-M1 isolate (table 1).

Animals captured and serologic assays. Two species of small mammals, rats (*Rattus norvegicus*) and mice, were captured in February and October 1988 in Pozarevac. In February, capture rates were 20% for rats (12 in 60 traps) and 40% (8/20) for mice. Of 16 rats and 21 mice trapped in February and October 1988, hantavirus antigen was found by IFA in lungs of 10 each (63% of rats, 48% of mice). Sera from all 16 rats and 6 mice (29%) had anti-hantavirus antibodies by IFA.

Virus isolation. Four pools of lung tissues from antigen-positive mice were processed for virus isolation. At 28 days after inoculation, 3 of 5 adult rats inoculated with mouse

tissues collected in February 1988 developed specific hantavirus antibody, and hantavirus antigen was found in lung tissue of 2 of 3 rats. The growth of newborn rats inoculated ic and ip was impaired, and they died 2 weeks after inoculation. Viral antigen was found in brain tissues of all 5 newborns. Intracytoplasmic, virus-specific, granular fluorescence was detected by IFA in Vero E-6 cells after two blind passages with convalescent serum from the adult Pozarevac patient and rabbit sera immune to HTN and PUU viruses. This isolate was POZ-M1. Similar results were obtained from a second pool of mouse tissues collected at the same locality but in October 1988. This isolate was not fully characterized serologically, although it appeared to be similar or identical to POZ-M1 on preliminary testing.

Laboratory rats inoculated with lung suspensions from 12 rats captured at the outbreak site were similarly processed, and 5 developed antibodies against both HTN and PUU viruses, with higher titers to the latter. Total cellular RNA prepared from Vero E6 cells infected with this material was amplified by PCR with an S-segment hantavirus-reactive primer pair (data not shown); however, we were unable to adapt it to growth in cell cultures, and it was eventually lost without further characterization.

Characterization of POZ-M1. The virus isolated from mice was characterized by cross-IFA, -EIA, and -PRN tests. High titers were measured by IFA when rat sera immune to POZ-M1 and PUU virus were tested with either the POZ-M1 isolate or PUU virus (table 1). Similar reactions were seen in cross-EIA tests (table 1). The isolate was compared with HTN and PUU viruses against the patient's convalescent sera and homologous antisera in cross-PRN (table 1), and POZ-M1 reacted to equivalent titers with both the patient's sera and immune rat sera against POZ-M1 or PUU. Thus, serology, including PRN, showed that POZ-M1 is an isolate of PUU virus, closely related or identical to the one that caused severe HFRS in the 46-year-old man.

RT-PCR and restriction endonuclease analysis. POZ-M1 was amplified with both M- and S-segment genus-reactive

primer pairs (data not shown). Restriction patterns of both products were identical to those of Hallnas-B1, a PUU virus isolated from bank voles (*Clethrionomys glareolus*) captured in Scandinavia (figure 1). These genetic characteristics further confirm that POZ-M1 is an isolate of PUU virus.

Discussion

During the epidemiologic investigation of the February 1988 outbreak, rodents were abundant in and around the house of the infected children. Only rats and mice were captured, and the common maintenance hosts of hantaviruses in Yugoslavia, *Apodemus flavicollis* and *C. glareolus*, were not encountered. All of the rats and 29% of the mice had anti-hantavirus antibody on IFA. Hantavirus antigen was also found in lung preparations of both species. These results clearly indicate that the domestic rodent populations were heavily infected with a local strain of hantavirus and were likely the sources of the human illness.

Previous investigations of HFRS found hantavirus antigen in the lungs of *M. musculus* captured in Serbia during 1981–1983 [7], when hantavirus antigen was found in lung tissues from 14% of mice by IFA. The isolation reported here represents the first recovery of a hantavirus from house mice captured in Yugoslavia.

The strain of PUU virus isolated, POZ-M1, was serologically associated with the severe case of HFRS. Although fatal HFRS due to PUU virus has been reported [15], PUU virus is generally associated with a much milder form of HFRS, nephropathia epidemica. Additional studies will be required to determine if POZ-M1 differs genetically from other PUU isolates.

At present, three genetic variants have been described among PUU viruses [2]: Sotkamo, CG18-20 (including isolates P360 and K-27), and Hallnas-B1 (including isolates Vranica and Leakey). Restriction pattern comparisons reported here indicate that POZ-M1 is closely related to Hallnas-B1. Among other isolates resembling Hallnas-B1, Lea-

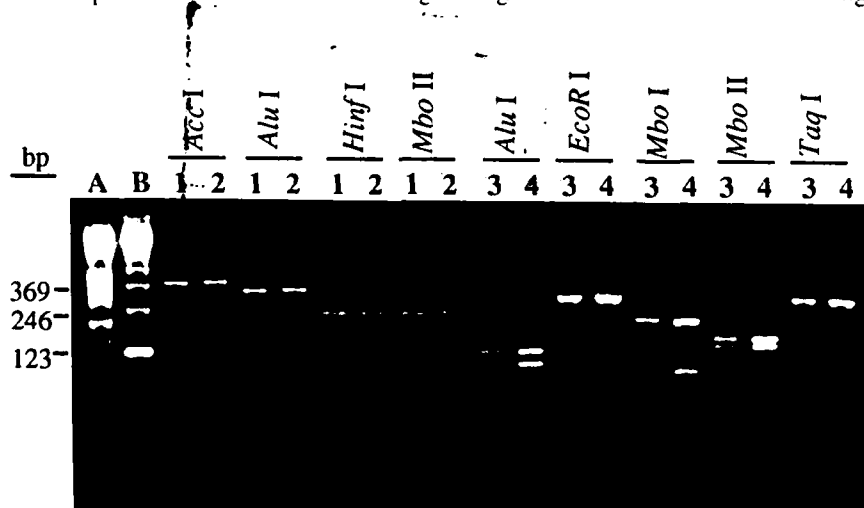


Figure 1. Comparison of polymerase chain reaction amplicons from POZ-M1 and Puumala virus strain Hallnas-B1 by restriction fragment length polymorphism. Lanes 1 and 2, M-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 3 and 4, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 5 and 6, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 7 and 8, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 9 and 10, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 11 and 12, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively. Lanes 13 and 14, S-segment amplicon of POZ-M1 and Hallnas-B1, respectively.

key was also recovered from *M. musculus*, but in the United States, whereas Vranica was isolated from *C. glareolus* trapped in Yugoslavia [8]. Isolation of POZ-M1 and demonstration of its reactivity with convalescent sera from a patient with associated severe HFRS suggest that this subgroup of PUU viruses may be a significant cause of human morbidity and mortality from HFRS in Yugoslavia and perhaps elsewhere. Recovery of PUU viruses from both *C. glareolus* and *M. musculus* indicates that previously proposed host-virus relationships may not be as strict as expected. In addition, it seems that PUU viruses may be yet another subgroup of hantaviruses with a very broad geographic distribution.

Hantaviruses have previously been associated with infections in laboratory rats that resulted in acute HFRS and death among animal handlers and others exposed to infected rodent colonies [3]. Investigations of past laboratory animal-associated outbreaks generally found only rats infected with hantaviruses; however, results presented here clearly demonstrate that *M. musculus* can harbor pathogenic hantaviruses as well. This observation adds credence to the concern that *Mus*-associated hantaviruses might also represent a potential hazard in the laboratory. Thus, it seems prudent to include hantavirus screening in routine quality control procedures for both laboratory rats and mice.

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